DISCERNING REGULATORY CONTROL OF PICROSIDES BIOSYNTHESIS IN A MEDICINAL HERB, *PICRORHIZA KURROA* ROYLE EX. BENTH

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DISCERNING REGULATORY CONTROL OF PICROSIDES BIOSYNTHESIS IN A MEDICINAL HERB, *PICRORHIZA KURROA* ROYLE EX. BENTH

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Introduction

Picrorhiza kurroa Royle ex. Benth is an important medicinal herb of Schrophulariaceae family found in alpine Himalayan range at an altitude of 2700-4500 m. Popularly, known as 'Kutki' it forms a major ingredient of traditional as well as modern medicine in India, China, Tibet and Nepal [1]. Iridoid glycosides, picroside-I and picroside-II (P-I and P-II) are principle active constituents derived from this herb and are responsible for several hepatoprotective, anti-inflammatory, immunomodulatory and anti-oxidant properties [2]. Herbal drugs like Livocare, Kutaki, Livomap, Picroliv, Katuki, Livplus, Pravekliv, Livomyn, and Vimliv [2] are prepared using *P. kurroa*. It has been reported that P-I and P-II biosynthesis occurs in shoots and roots, respectively while stolons and rhizomes accumulate both the picrosides [2, 3]. Underground parts of the plant like roots and rhizomes make this plant valuable to the industry (Fig. 1).

Figure 1. Different organs of a mature *P. kurroa* plant

Narrow range of distribution, low seed viability along with overexploitation of natural habitat have placed this plant under endangered species listed by International Union for Conservation of Nature and Natural Resources and restricted its trade across national territories [4]. For alleviating the endangered status of this medicinal species, *in vitro* propagation has been successfully implemented but maintenance of phytochemical identity under *in vitro* conditions remains a major issue. Several studies have attempted enhancement of P-I content *in vitro* via media optimization [3], application of different precursors and natural as well as synthetic elicitors [5, 6] but significant increase in P-I content has not been achieved. Moreover, limited production of P-I in shoot cultures compared to natural habitat indicates involvement of several environmental factors apart from temperature, in affecting P-I biosynthesis.

Recent elucidation of biosynthetic pathway for picrosides provided a basic understanding of picrosides production in *P. kurroa* [7]. Their biosynthesis is a complex event involving multiple pathways like non-mevalonate (MEP), mevalonate (MVA), phenylpropanoid and iridoid pathways (Fig.2) which are connected in a regulated cross-talk, ultimately resulting in P-I and P-II production. In addition to pathway elucidation, previous studies have also emphasized the importance of several genes like *ISPD, DXPS, ISPE, PMK, 2HFD, EPSPS, SK, HMGR, DAHPS, PAL, CM, GS* and *G10H* in controlling flux of individual pathways in picrosides production [7, 8, 9]. Although biosynthetic machinery has been extensively explored, no information exists regarding regulatory control of biosynthetic pathway or processes associated with picrosides production in *P. kurroa*. Targeting individual pathway genes as candidates for genetic engineering approaches such as silencing, overexpression and generation of knockouts would be cumbersome as a total of 42 genes constitute the complete biosynthetic pathway. Moreover, pathway genes which have been highlighted for their significant role in controlling picrosides accumulation belong to different integrating pathways of picrosides production. In such a scenario, identification of regulatory genes that control multiple pathway genes would prove ideal candidates for pathway engineering.

The most common and explicit form of gene regulation is exercised by transcription factors which have been known to control transcription of genes under the effect of developmental stage, tissue specificity and environmental conditions via binding to corresponding DNA binding domains in their promoter regions [10]. Role of TFs as master regulators of gene expression has been depicted in *Catharanthus roseus* where overexpression of transcription factor ORCA3 resulted in synchronized elevation of five TIA biosynthetic pathway genes [10]. As picrosides biosynthesis is modulated by various conditions like temperature, culture conditions, altitude and light response [3, 11, 12], analysis of transcriptional regulation of genes belonging to picrosides biosynthetic pathway along with processes relevant for picrosides biosynthesis would result in design of an optimal strategy for enhancing metabolites content.

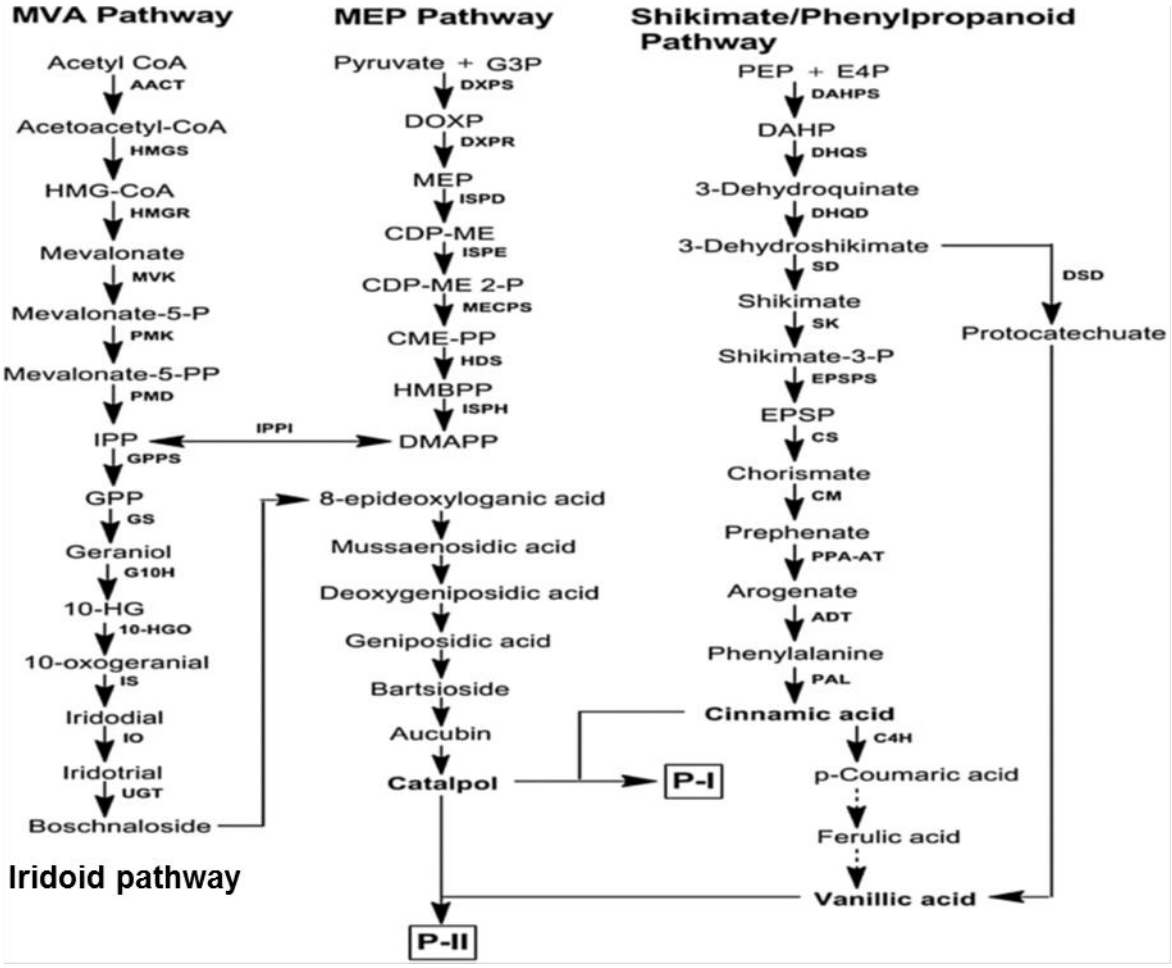

Figure 2. A representative diagram showing different modules of picrosides biosynthetic pathway in *P. kurroa* [6, 7]

miRNAs are another set of regulatory molecules popularly known for their pleiotropic nature of regulation [13]. These are endogenous 19-23 nucleotide non-coding RNAs that play vital roles in transcriptional and post-transcriptional regulation of gene expression. Recently, miRNAs have been highlighted as new potential bioactive compounds in medicinal plants due to properties like transferability across species [13]. Exploration of their role in regulation of secondary metabolism has gained significant attention recently. miR393 has been known to modulate levels of glucosinolate and camalexin in Arabidopsis and several miRNAs like pso-miR2161, pso-miR13 and pso-miR408 have also been identified as potential regulators of alkaloid biosynthesis in poppy [14, 15]. Due to combinatorial nature of miRNAs, their role in down regulation of several processes relevant for picrosides biosynthesis via target degradation can be speculated in tissue cultured conditions of *P. kurroa*.

It has been observed that under *in vitro* conditions, if all the factors associated with tissue culture like source explant, culture conditions and media constituents are kept same but incubation temperature is varied, significant variation in shoot biomass (~ 2 folds) and P-I content (~ 60

folds) is observed between shoot cultures (PKS-15- 0.6% and PKS-25- 0.01%) of *P. kurroa* incubated at 15° C (PKS-15) and 25° C (PKS-25) [16]. On the other hand, in plants obtained from natural habitat (PKSS), the P-I content increases up to 2.7% which is considerably higher than *in-vitro* cultured plants. It is clearly indicative that certain physiological processes get altered under varying environmental conditions thereby affecting P-I content. No attempts have been made yet at identifying molecular components that perceive signals under differential conditions of growth and elicit P-I production. Identification of such signals that act in coherence with P-I production would aid in understanding mechanisms possibly leading to low metabolite production *in vitro*. Moreover, leaves are the primary sites for P-I biosynthesis [2, 4] but limited efforts have been made at understanding molecular basis of shoot biomass productivity which is a major contributing factor associated with their biosynthesis.

Previous studies have revealed that phosphorylation mediated by protein kinases is a key signal transduction mechanism utilized by eukaryotes for regulating almost all activities which define their phenotype [17]. Several reports have highlighted the role of kinases in mediating defense against environmental factors as well as elicitation of secondary metabolism in plant species. For example, *AtCPK1* has been observed to mimic impact of elicitors on anthraquinone (AQ) production via activation of ROS in *Rubia cordifolia* cells [18]. MAP kinases like MPK3 and MPK4 have also been known to affect monoterpenoid indole alkaloids and camalexin biosynthesis in *Catharanthus roseus* and Arabidopsis, respectively [19, 20]. Therefore, analysis of signal transduction kinases can result in identifying processes that perceive differential environmental conditions and stimulate picrosides accumulation in *P. kurroa*.

Keeping in view the gaps existing in regulatory control of picrosides biosynthesis as well as no information on kinases, the current study was undertaken with the following objectives:

Objective 1: Discerning the role of transcription factors and miRNAs in controlling the biosynthesis of picrosides in *P. kurroa*

Objective 2: Mining differential transcriptomes of *P. kurroa* shoots for kinases and associating their possible role in signal perception for picroside-I production.

MATERIALS AND METHODS

Plant Material

Three *Picrorhiza kurroa* Royle ex. Benth organs obtained from natural habitat as well as *in vitro* conditions were chosen for transcriptome mining and gene expression analysis. The shoot samples consisted of two *in vitro*-cultured conditions maintained at 25 °C and 15 °C (PKS-25, PKS-15) temperature controlled chambers in Jaypee University of Information Technology, Solan, Himachal Pradesh (1,544 m, 31.02°N, 77.08°E) and field-grown shoots (PKSS- natural habitat) obtained from Sairopa, Himachal Pradesh (4,500 m, 31°380–31°540N and 77°200–77°450E). Root samples included *in vitro*-cultured roots (PKR-25) maintained at 25 °C

temperature controlled chambers and field-grown roots (PKSR- natural habitat). Stolons were procured from field-grown plants of Sairopa (PKSTS). *In vitro* cultured plants were cultured on MS media [5] supplemented with indole-3-butyric acid (IBA-3 mg/l), sucrose (30 g/l), kinetin (1 mg/l) and agar (9 g/l) and pH of media was adjusted to 5.7. The 16 h photoperiod was provided by cool white fluorescent light (3000 lux Philips, India) and 70% humidity was maintained in the tissue culture chamber. High (PKS-1- 2.7 %) and low (PKS-4- 0.3 %) P-I content strains of *P. kurroa* were procured from greenhouse of Jaypee University of Information Technology.

Mining for miRNAs and their targets in transcriptomes from roots, shoots and stolons of *P. kurroa*

All available plant miRNAs and their fold back sequences obtained from miRbase (http://www.miRbase.org/) were used as a reference dataset for mining conserved miRNAs in six transcriptomes (PKS-15, PKS-25, PKSS, PKR-25, PKSR and PKSTS) of *P. kurroa* [21] using in-house developed PERL script, followed by their secondary structure prediction by Mfold software (http://unafold.rna.albany.edu/?q=mfold). Corresponding targets for these miRNAs were predicted using psRNA Target (A Plant Small RNA Target Analysis Server) (http://plantgrn.noble.org/psRNATarget/). Functional annotation of miRNA targets was explored in NCBI-NR database using default parameters of BLASTX. Widely used databases like GO, COG and KEGG were mined for linkage of miRNAs with corresponding physiological processes using default parameters.

5' RACE-mediated miRNA target cleavage validation

To check for miRNA-directed cleavage, First Choice RLM-RACE kit (Ambion) was used in a 5'RACE experiment following manufacturer's directions. Gene-specific outer and inner primers were used for nested PCR analysis. The products of second nested PCR were gel purified (Promega) and cloned using pGEMT easy (Promega) plasmid kit and were subsequently sequenced on 3500XL Gene Analyzer (Applied Biosystems).

Mining of genes in root and shoot transcriptomes of *P. kurroa*

In-house assembled transcriptomes of five *P. kurroa* tissues (PKSR, PKR-25, PKSS, PKS-25, PKS-15) (available at http://14.139.240.55/NGS/download.php) [21] were mined for identification and classification of TFs using in-house developed PERL scripts and BLASTX search against NR database. The annotation of transcripts was further verified via search against PlantTFcat (http://plantgrn.noble.org/PlantTFcat/familylist.do) with an e-value cutoff of 1e−05 and classified into TF families. Out of these families, transcripts encoding members of WRKY, MYB, NAC, Zinc Finger, bHLH, bZIP and AP2/ERF transcription families were chosen for further analysis.

For identification of different types of kinases, shoot transcriptomes (PKSS, PKS-25 and PKS-15) were explored using in-house developed PERL scripts by performing BLASTX with NR (non-redundant) database with an E-value threshold 1e-05. All the results were checked manually to avoid any error. Nucleotide sequences for genes implicated in shoot biomass productivity like *AXR-1, GRF-2, AVP-1, CLAVATA-1, TOR-1, AUX-1, LAX, DA-1* and *ARF-2* were retrieved from the GenBank and were searched in shoot transcriptomes (PKS-25 and PKS-15) using similarity search.

Identification of common and unique transcripts for TFs and kinases

Among shoot transcriptomes (PKS-25, PKS-15 and PKSS), all the common transcripts encoding selected TF families, kinases and shoot biomass genes were shortlisted. Common as well as unique TF transcripts were shortlisted between root tissue transcriptomes, (PKR-25 and PKSR). For comparative analysis, BLASTN was used for homology search between transcriptomes with a cut-off E-value threshold 1e−06 and the results were verified manually.

Transcript abundance of candidate transcription factors, kinases and shoot biomass genes

RNA-Seq by Expectation Maximization (RSEM) approach was used for quantification of transcript abundance from the RNA-Seq high-quality data. Default parameters were used for transcript quantification of TFs and kinases for *de novo* assembled sequences. Common TFs and kinases present in all three shoots (PKS-25, PKS-15 and PKSS) and roots (PKR-25 and PKSR) were selected independently and their transcript abundance was quantified. The fold variation was calculated in high versus low P-I/P-II content tissues. In shoot datasets, common TF transcripts and kinases showing more than two fold and more than five-fold higher abundance (FPKM) in PKS-15 and PKSS, respectively, in comparison to PKS-25 were selected. In addition, TF transcripts present in PKS-15 and PKSS showing lower abundance than PKS-25 were also selected. In root datasets, TFs showing more than two-fold higher abundance in PKSR with respect to PKR-25 along with TF transcripts present in PKSR showing lower abundance than PKR-25 were selected. Shoot biomass genes common to PKS-15 and PKS-25 having higher transcript abundance in PKS-15 versus PKS-25 were selected.

***In silico* analysis of promoter regions**

Several studies aimed at understanding the biosynthesis of picrosides in *P. kurroa* have highlighted the role of *ISPD, DXPS, ISPE, PMK, 2HFD, EPSPS, SK, HMGR, DAHPS, PAL, CM, GS* and *G10H* as key genes involved in the biosynthesis of picrosides [7, 8, 9]. To study the effect of TFs analyzed in our study on these key genes, TF binding sites were explored in their promoter regions. However, only a few promoter regions were available for picrosides biosynthetic pathway genes (*ISPE, DXPS* and *HMGR*) as genome of *P. kurroa* has not been sequenced. For analysis of remaining gene promoter regions, their dicot orthologs were extracted from the Genbank and analyzed. No promoter regions were available for genes *PMK, 2HFD,*

DAHPS, *SK* and *CM* in the Genbank. Promoter regions for *ISPD*, *EPSPS*, *GS*, *G10H* and *PAL* genes were retrieved from dicot species like *Stevia rebaudiana*, *Convolvulus arvensis*, *Catharanthus roseus* and *Camelia sinensis*. The promoter regions were analyzed for sequence elements by using PLACE (http://www.dna.affrc.go.jp/PLACE) and JASPAR Core (Plant) database (http://jaspar.genereg.net/) with default parameters. For each matrix obtained for TF binding sites in JASPAR, PSSM matches with a cutoff score above 5.0 were considered as possible binding sites.

Isolation of RNA and cDNA synthesis

Total RNA was isolated from *P. kurroa* tissues (100mg) using TRIzol reagent (Invitrogen) and digested with DNaseI (Invitrogen). First-strand complementary DNA synthesis (cDNA) was perfomed using Verso cDNA synthesis kit (Thermo Scientific, USA) from total RNA template as per manufacturer's instructions. cDNA preparation from miRNA was carried out using NCode™ miRNA First-Strand cDNA Synthesis (Life technologies) by selective poly-A tailing of miRNA and subsequent cDNA synthesis from poly-A miRNAs. The primers for qRT-PCR analysis were designed using Primer 3 v.0.4.0 software (http://bioinfo.ut.ee/primer3-0.4.0/).

Expression analysis using quantitative real time PCR (qRT-PCR)

Equal quantities of cDNA (100ng) of each tissue sample were taken for analyzing gene expression levels. Conventional PCR was used for amplification of a single product using gene specific primers. qRT-PCR reaction was performed using SYBR Green PCR master mix (Bio-Rad Laboratories) in a CFX96 system (Bio-Rad Laboratories; Hercules CA, USA). All reactions were performed in three technical replicates and repeated twice. Comparative Ct method was used for evaluation of relative expression of each gene. 26s, elf-1α and 5.8s were used as internal controls for normalization of gene expression.

Identification of causal agent of leaf blight in *P. kurroa*

During sample collection in natural habitat, small, circular, brown necrotic spots were observed on the abaxial and adaxial sides of the leaves of *P. kurroa*. For identifying the causal agent, the pathogen was isolated, cultured and observed under BX53 fluorescent microscope (Olympus). Molecular characterization was carried out by amplifying a portion of ITS1/4 region, followed by species validation via species specific primers. Corresponding amplicons were subsequently cloned and sequenced (3600xl Gene Analyzer, Applied Biosystems). In addition, Koch's postulates were conducted to prove pathogenicity by inoculating twelve detached, healthy leaves with 5 µl (1×10^5 spores/ml) conidia suspension which were incubated in a growth chamber with 80-90% relative humidity and a 12h photoperiod.

RESULTS AND DISCUSSION

Identification of miRNAs and their targets in transcriptomes of shoots, roots and stolons in *P. kurroa*

Transcriptome mining of shoots (PKS-25, PKS-15, PKSS), roots (PKR-25, PKSR) and stolons (PKSTS) using miRbase as a reference database, revealed 18 miRNAs in *P. kurroa* which have been evolutionarily conserved across several monocots, dicots and eudicots. These miRNAs belonged to 18 families and length of the sequences ranged from 18 to 22 nucleotides and the sequences were a perfect match (100%) to the corresponding homologs in other species. miRNAs express their entity through target binding, so knowledge regarding the complementary mRNA sequence becomes crucial in order to understand the effect as well as the mechanism of action of these molecules. psRNA target prediction server identified a total of 30 targets for 18 miRNAs having near-perfect complementarities. The study predicted several novel functions for miRNAs in *P. kurroa* which reflects that at least some conserved miRNAs are regulating new targets in addition to the well-documented conserved targets in other plant species. Various transcription factors like ethylene responsive, basic loop helix loop, squamosal promoter-binding-like protein, leucine-rich repeats, etc. along with multiple kinases and phosphatases were identified as targets of miRNAs. The potential targets were involved in several biological functions like signal transduction, nucleic acid metabolism, disease resistance, hormonal responses, transport, regulation, developmental processes as well as secondary metabolism. Target of miR-4995 was specifically interesting as was found to encode an enzyme 3-deoxy-7-phosphoheptulonate synthase (DAHPS) of shikimic acid pathway, a major contributing pathway involved picrosides biosynthesis.

Expression analysis of miRNAs by qRT-PCR

Expression analysis of conserved miRNAs in tissues of *P. kurroa* revealed that stolon (PKSTS) had a higher expression profile for maximum (39%) miRNAs and field-grown shoot (PKSS) had lowest expression for almost all miRNAs. *In vitro* roots had higher expression for 28 % of miRNAs and *in vitro* shoots PKS-15, PKS-25 had higher expression for 5 % and 11 % of miRNAs, respectively. In case of root as well as shoot tissues, *in vitro*-cultured samples showed a higher miRNA expression profile as compared to field grown samples. Such an observation is not obvious, but it has been reported that *in vitro* culture environment provides conditions for various epigenetic modifications like rejuvenation in herbaceous and woody plants [22]. Epigenetic mechanisms have been reported to alter miRNA expression [23] which might be the possible reason for the observed expression pattern of miRNAs in *P. kurroa*.

Role of miRNAs in controlling picrosides accumulation in *P. kurroa*

Terpenoids are generally produced by two metabolic pathways, mevalonate (MVA) and non-mevalonate (MEP). Isopentenyl pyrophosphate (IPP) and dimethyl allyl pyrophosphate

(DMAPP) are end products of both the pathways and these condense to form geranyl diphosphate (GPP), the precursors of monoterpenes. GPP leads to the formation of catalpol which combines with cinnmate or vanillate moieties derived from phenypropanoid/shikimic acid pathway to produce picrosides [7, 24] (Fig.3(a)). miR-4995 was observed to target mRNA encoding 3-deoxy-7-phosphoheptulonate synthase, an enzyme involved in the first step of phenylpropanoid pathway which contributes to the production of cinnamic acid and vanillic acid. Being the first enzyme of the pathway, its down-regulation can affect the flux of pathway, resulting in low P-I content. miR-4995 was present in all three shoot samples, with maximum expression in PKS-25 which has negligible P-I content, whereas least expression was observed in PKSS which has maximum P-I content. For confirmation of down-regulatory effect of miR-4995, expression quantification of target mRNA transcript encoding 3-deoxy-7-phosphoheptulonate synthase was analyzed by qRT-PCR in all three shoot tissues (Fig. 3(b)).

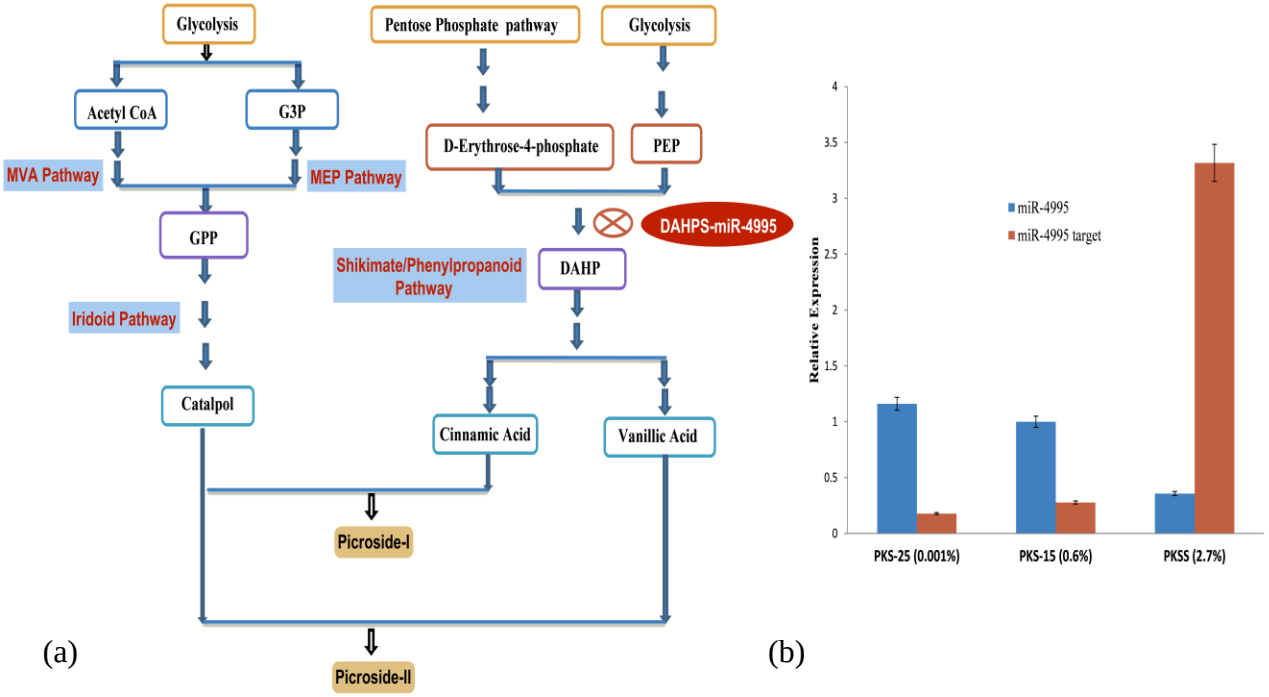

Figure 3. (a) Depiction of miRNA-4995 and corresponding target in picrosides biosynthetic pathway (b) Gene expression analysis showing negative correlation of miR-4995 and target transcript in *in vitro* cultured shoots (PKS-25, PKS-15) and field grown shoots (PKSS).

Field-grown shoot (PKSS) had ~10 fold higher expression of DAHPS as compared to *in vitro* cultured shoots. As P-I is produced in all three shoots, this enzyme is also present but the complementary mRNA sequence (21 nucleotides) for miR-4995 was found only in low P-I content tissue cultured samples (PKS-25 and PKS-15). Absence of the target in PKSS could result in prevention of regulation by miRNAs, leading to higher biosynthesis of P-I as compared to tissue cultured counterparts. Expression pattern of miR-4995 in shoot samples was PKS-25 >

PKS-15 > PKSS, whereas the target mRNA showed an opposite expression pattern PKSS > PKS-15 > PKS-25 thereby establishing a negative correlation between miRNA and its target mRNA. It can be inferred that miR-4995 lowers the expression of DAHPS by pairing with the complementary sequence, resulting in cleavage of the transcript.

Further, to validate cleavage-mediated target degradation, modified 5'-RLM RACE was performed on RNA extracted from PKS-25, PKS-15, and PKSS. PKS-25 and PKS-15 showed near-perfect complementarity between miR-4995 and corresponding mRNA target, thereby establishing target down-regulation by miRNA : mRNA base pairing (Fig. 4). Roots also showed a major variation in miRNA-4995 profile with field-grown roots (PKSR) showing minimal miRNA expression as compared to tissue cultured roots (PKR-25). PKSR has considerably high P-II content, whereas PKR-25 do not produce P-II but significant correlation between P-II content and miRNA-4995 could not be established as the target sequence for miR-4995 was absent in both root tissues (PKSR and PKR-25).

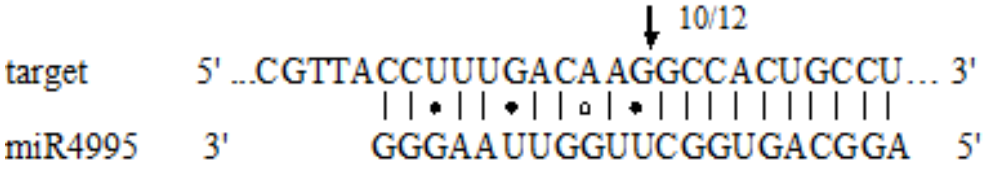

Figure 4. miRNA sequence along with target mRNA transcript sequence. Watson–Crick base pairs are indicated with vertical dashes, G–U base pairs are indicated with a bold dot and mismatch with an empty dot. The arrow indicates the cleavage sites of target region and the numbers show the frequency of clones that started from the position.

Identification of transcription factors associated with secondary metabolism in roots and shoots of *P. kurroa*

TFs belonging to families which have been previously implicated for secondary metabolism in plant species [25] were mined in *P. kurroa* root and shoot transcriptomes. In root samples, 82 and 101 transcripts encoding TF families were identified in PKR-25 and PKSR, respectively. Similarly, in shoot samples, 131, 137 and 107 transcripts encoding these TFs were identified in PKS-25, PKS-15, and PKSS, respectively. *In vitro* cultured shoots (PKS-25 and PKS-15) had an abundance of MYB family whereas shoots obtained from natural habitat (PKSS) had a predominance of WRKY family. Similarly, *in vitro* cultured roots (PKR-25) had maximum members of NAC and MYB families and roots obtained from natural habitat had maximum members belonging to WRKY family (Fig. 5).

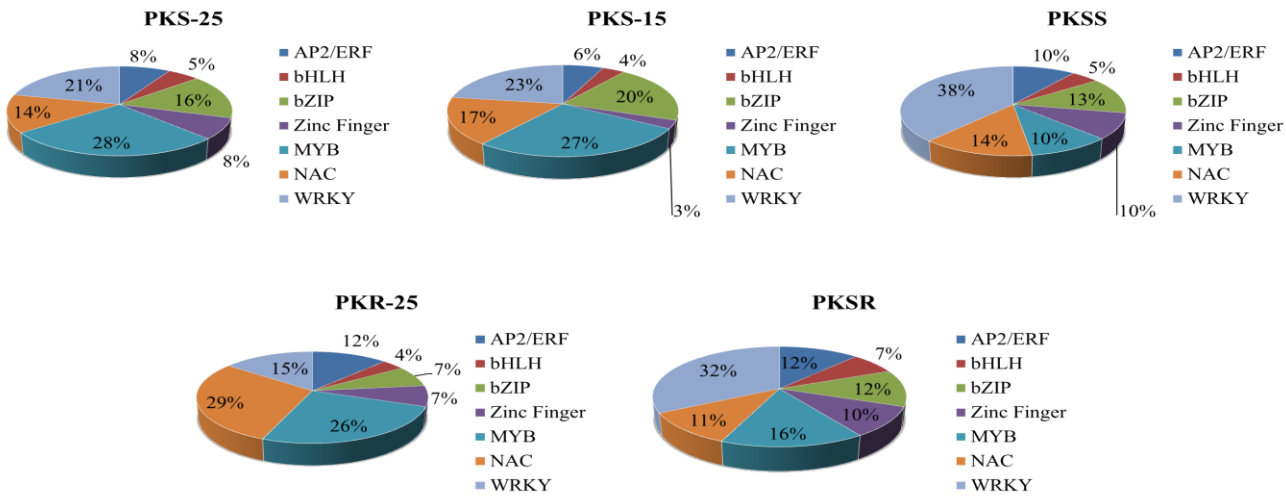

Figure 5. Proportionate distribution of TF families in shoots and root transcriptomes of *P.kurroa*

Identification of unique and common transcription factors and prediction of their transcript abundance

A total of 77 TF transcripts were found to be common to all three shoot tissues out of which 36 TFs were selected for analysis via qRT-PCR based on transcript abundance. AP2 domain TF, ERF-(109, 18, 32, 34, 118), bHLH-(80, 104), bZIP-(5, 60, gbf-3), MYB-(3, 28, 108, 96), NAC-(14, 25, 32, 94, NAP, SNAC-1), WRKY-(20, 5, 27, 41, pkdoubleWRKY) and Zinc Finger (SUF-4, COL-10, Nutcracker) showed progressive increase in transcript abundance in the trend PKS-25 < PKS-15 < PKSS, whereas transcripts corresponding to bHLH-93, MYB-4, NAC-22 and WRKY-34, 40, 17 and 71 had abundance in the opposite trend i.e. PKS-25 > PKS-15 > PKSS. In root tissues, total of 56 TF transcripts were found to be common to both PKSR and PKR-25. Out of these, 41 TFs were selected for validation via qRT-PCR analysis. AP2, ERF-(21, 18, 113), bHLH-(93, 104), bZIP-(5, CPRF- 2), MYB-(3, 5, 6, 96), NAC-(14, 22, 25, 32, 94, SNAC-1), WRKY-(5, 20, 34, 41, pkWRKY, pkdoubleWRKY) and Zinc finger (SUF-4, COL-7 and Nutcracker) TFs had more than two fold higher abundance in PKSR with respect to PKR- 25 whereas, ERF-(109, 32, 34, 118), bHLH-80, bZIP- 60, MYB-(4, 28), NAP, WRKY-(40, 17, 27, 71) and Zinc finger (COL-10) had higher abundance in PKR-25 than PKSR. MYB-108 and gbf TFs, known to be responsive to Jasmonate [26] and light [27] respectively, were found to be unique to PKR-25. *In vitro* cultured *P. kurroa* plant roots are exposed to a 12 h photoperiod unlike underground field roots, so negligible expression of gbf in PKSR may be due to absence of light exposure. However, reason behind exclusive expression of MYB-108 in PKR-25 is unclear.

Expression analysis of transcription factors in shoot tissues via qRT PCR

Out of 36 TFs analyzed in shoot tissues (PKS-25, PKS-15 and PKSS), 16 TFs showed higher expression in PKS-15 as compared to PKS-25. Out of these, five TFs i.e. AP2, MYB-3, 96,

SNAC-1 and pkdouble WRKY which had shown higher expression in PKS-15 versus PKS-25, did not show higher expression in PKSS compared to PKS-15, indicating that these are probably just temperature responsive under *in vitro* culture conditions and might not be affecting picroside-I biosynthesis. Exposure to abiotic stress like fluctuation in temperature conditions is known to cause oxidative stress coupled with release of several reactive oxygen species (ROS) and plants often employ several enzymatic and non-enzymatic ways of dealing with the stress [28] which may be under the influence of such TFs. 11 TF's namely ERF-(18, 118), bHLH-80, 104, bZIP-60, MYB-28, NAC-(25, 32, 94, NAP) and SUF-4 showed a progressive increase in transcript abundance in the order of PKS-25 < PKS-15 < PKSS, thereby following the trend of picroside-I content in shoots of *P. kurroa*. Elevation of these TFs in natural habitat conditions as compared to *in vitro* shoots might be a biochemical adaptation response to several factors like UV radiation, plant-pathogen interaction and abiotic stress conditions along with elicitation of picrosides biosynthesis. To ascertain TFs which are specifically associated with picrosides biosynthesis, variation in culture conditions was removed by analyzing expression of these TFs in high (PKS-1- 2.7 %) versus low (PKS-4- 0.3 %) P-I content genotypes. These genotypes had been grown under uniform greenhouse conditions and varied only for P-I content. It was observed that all these TFs had elevated expression in high P-I content genotype too (Fig. 6). bHLH-80, NAC-25, 32 and NAP showed a substantial (>15 fold) elevation in expression in high versus low P-I genotype indicating their predominant role in affecting P-I content between these genotypes. TF's like MYB-4, NAC-22 and WRKY-(34, 40, 17, 71) showed a progressive decrease in expression in the trend PKS-25 > PKS-15 > PKSS, indicating their possible role in down regulating P-I biosynthesis in tissue cultured shoots.

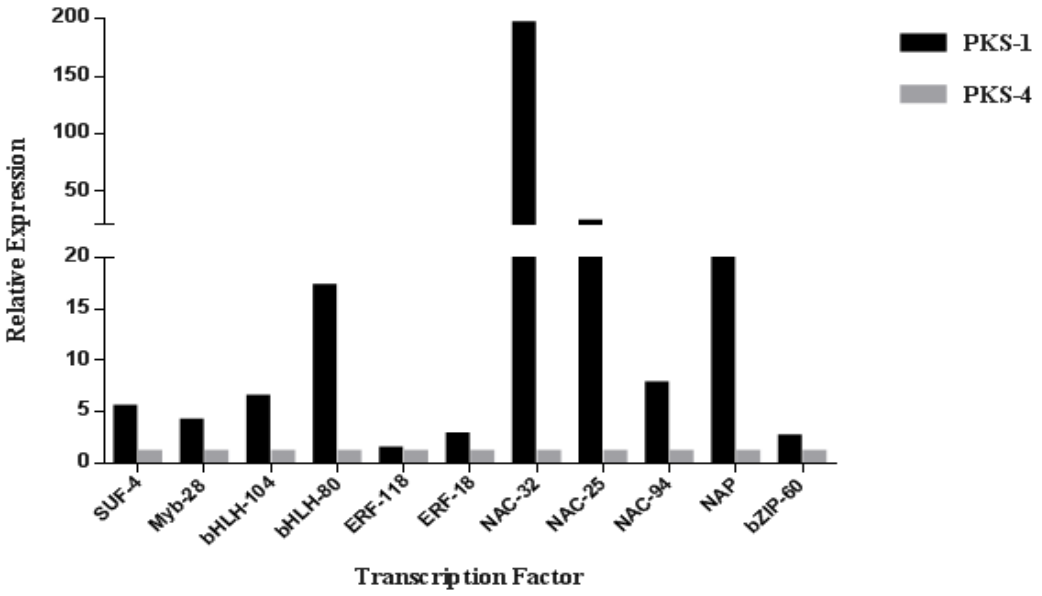

Figure 6. Relative expression of transcription factors in high (PKS-1- 2.7 %) versus low (PKS-4- 0.3%) P-I content shoot genotypes of *P. kurroa*

Expression analysis of transcription factors in root tissues via qRT PCR

In root tissues, it was observed that most of the TF transcripts showed higher expression in P-II producing roots (PKSR) as compared to non P-II producing roots (PKR-25) (Fig. 7). Such a pattern of expression variation can be due to differential conditions encountered by these root variants like abiotic/biotic stress, root morphology and culture conditions which could be a major activator of TFs expression.

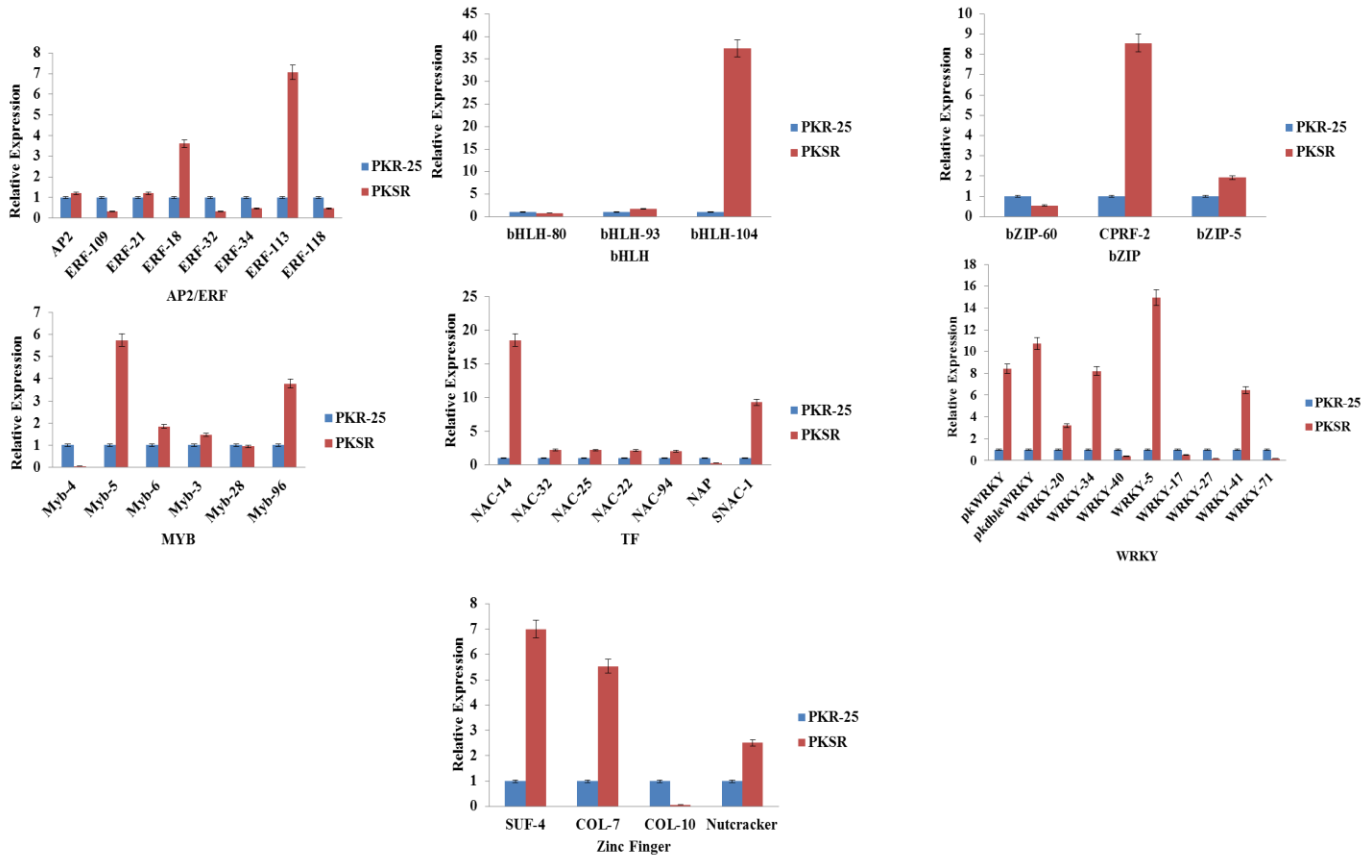

Figure 7. Relative expression of TFs between *in vitro* cultured roots (PKR-25-0 %) and roots obtained from natural habitat (PKSR- 0.4 %). Error bars correspond to percentage error (5 %)

Association of TFs with Picroside-I and Picroside-II accumulation in *P. kurroa*

As P-I and P-II biosynthesis occurs via a common biosynthetic route, identification of commonly up regulated TFs among high picrosides producing roots and shoots of natural habitat conditions would give clues regarding their regulatory role in picrosides accumulation. TFs showing expression variation vis-a-vis picrosides content in natural habitat variants of roots as well as shoots included ERF-18, bHLH-104, NAC-25, 32, 94 and SUF-4, indicating their possible role as activators of picrosides biosynthesis in *P. kurroa*. In our study, members of NAC family were observed to play a significant role in regulating picrosides biosynthesis in *P. kurroa* as

expression of multiple members (NAC-25, 32 and 94) was positively correlated with picroside-I and II content. NAC TFs have been reported to play a major role in monoterpene synthesis in kiwifruit [29], thus their involvement in biosynthesis of picrosides which are monoterpenoids in nature, can also be expected. Elevated expression of TFs like WRKY-17, 40, 71 and MYB-4 in low P-I producing *in vitro* cultured shoots (PKS- 25 and PKS-15) and non P-II producing *in vitro* cultured roots (PKR-25) in comparison to natural habitat conditions (PKSS and PKSR) implicates their potential role in suppression of processes associated with picrosides biosynthesis. These TFs are known transcriptional repressors of genes in plant species [30-33]. They appear to target enzymes which catalyze major control points of the contributing pathways, thus reducing the rate of individual pathway product formation, ultimately affecting picrosides production under *in vitro* culture conditions. Additionally, these TFs, under the control of environmental factors, might be affecting several molecular processes indirectly involved in the biosynthetic machinery of picrosides.

Detection of promoter sequence elements in picrosides biosynthetic pathway genes

In silico analysis of promoter regions of key genes involved in picrosides biosynthetic pathway (*HMGR, ISPE, DXPS, GS, G10H, EPSPS, ISPD* and *PAL*) revealed presence of potential binding sites for several members of TF families analyzed in the current study like MYB-(3, 4), WRKY-(40, 71), NAC-25, ERF-(18, 109) and bZIP-60 (Table. 1).

Table 1. Promoter analysis of key pathway genes and corresponding predicted TF binding sites.

S. No.	Promoter Sequence	Analyzed transcription factors having binding sites in promoter regions
1	*Picrorhiza kurroa HMGR* promoter region	MYB4, WRKY40, ERF18, WRKY71
2	*Picrorhiza kurroa IspE* promoter region	MYB3, MYB4, WRKY40, NAC25, ERF109
3	*Picrorhiza kurroa DXPS* promoter region	MYB3, MYB4, WRKY40, ERF109, bZIP60, NAC25, ERF18
4	*Camelia sinensis PAL* promoter region	NAC25, MYB3, MYB4, WRKY40, ERF18, ERF109
5	*Catharanthus roseus G10H* promoter region	WRKY40, NAC25, bZIP60, MYB4, ERF18
6	*Catharanthus roseus GS* promoter region	ERF18, WRKY40, MYB3, MYB4, NAC25, bZIP60,
7	*Convulvulus arvensis EPSPS* promoter region	MYB3, MYB4, ERF18, ERF109, WRKY40, NAC25,
8	*Stevia rebaudiana IspD* promoter region	MYB3, MYB4, NAC25, WRKY40,

A binding site for transcriptional repressors MYB-4 and WRKY-40 was observed in promoter regions of all key genes involved in picrosides biosynthesis. Their elevated expression in low picrosides producing tissue cultured plants indicates their possible involvement in down regulation of these genes under *in vitro* culture conditions. Role of MYB-4 in controlling phenylpropanoid pathway by regulating PAL gene has been reported in Arabidopsis [34]. PAL is a major regulatory enzyme of phenylpropanoid pathway which is an integrating pathway involved in picrosides biosynthesis (Fig. 2). Thus, MYB-4 is expected to modulate PAL gene expression in *P. kurroa*. Inhibitor experiments in *P. kurroa* have revealed that, out of all the pathways involved in picrosides biosynthesis, MEP is the major contributor for picrosides [7]. So, targeting TFs which are having binding sites in regulatory/rate limiting genes of this pathway (*ISPD, ISPE* and *DXPS*) coupled with their overexpression can modulate picrosides biosynthesis more efficiently. NAC- 25 and ERF-18 can be taken up as priority candidates as they appear to target all important key genes and their elevated expression under natural habitat conditions of roots as well as shoots points towards their possible involvement in activating picrosides biosynthesis. Approaches involving down-regulation/silencing of MYB-4 and WRKY-40 can be attempted for elevating picrosides content under *in vitro* culture conditions.

Identification of kinases in shoot transcriptomes varying for picroside-I content in *P. kurroa*

It has been widely accepted that phosphorylation mediated by protein kinases is a key signal transduction mechanism utilized by eukaryotes for regulating almost all activities which define their phenotype [17]. Therefore, it can be assumed that varying environmental signals are perceived by certain kinases and are further transduced to downstream machinery, ultimately affecting overall P-I yield in *P. kurroa*. Transcriptome mining in different shoots (PKS-25, PKS-15 and PKSS) of *P. kurroa* revealed multiple transcripts encoding various types of kinases involved in primary and secondary metabolism. A total of 521, 473 and 346 transcripts encoding kinases were identified in PKS-25, PKS-15 and PKSS transcriptomes, respectively. Among these, a total of 260 transcripts encoding kinases were found to be common in all shoot tissue transcriptomes. Further, *in silico* transcript abundance shortlisted 43 kinases which were observed to follow the trend of P-I accumulation among shoot variants (PKS-25(0.01%) < PKS-15(0.6%) < PKSS (2.7%)) and were taken up for gene expression analysis.

Expression analysis of kinases in *P. kurroa* shoots varying for Picroside-I

Several classes of kinases like receptor kinases/receptor like kinases, CDPKs, MAPKs and kinases involved in cellular processes were analyzed in tissue cultured and natural habitat shoot tissues. Upon comparison of *in vitro*-cultured shoots (PKS-15 versus PKS-25), most of the transcripts (33 out of 43) showed higher expression in PKS-15. Highest variation among tissue culture variants was observed in kinases involved in cellular processes (≤ 24 folds) followed by receptor kinases/RLKs having ≤ 6 folds variation, whereas MAPKs and CDPKs exhibited least

variation of ≤ 2 folds. These up regulated kinases have been reported to be involved in processes like amino acid and nucleotide metabolism, carbon fixation and carbohydrate metabolism, biotic/abiotic stress, hormonal response and organ development. Higher expression of these kinases in PKS-15 might be an adaptive response to growth under low temperature stress which might be affecting several metabolic processes including elicitation of secondary metabolism. To shortlist kinases relevant to P-I biosynthesis, these were further analyzed in natural habitat shoots (PKSS) which are grown at a similar temperature as PKS-15 but accumulate significantly higher P-I content. Screening of these up regulated kinases in PKSS versus PKS-15 shortlisted twenty-three kinases which showed a progressive increase in expression (PKS-25 < PKS-15 < PKSS), following the trend of P-I accumulation, indicating their possible role in affecting processes related to its accumulation. Among receptor kinases/receptor like kinases, most of the kinases showed an increase in expression in PKSS (≤ 16 folds) with respect to PKS-15. In case of MAPKs and CDPKs, a significant up regulation was observed in PKSS with respect to PKS-15 (≤ 51 folds and ≤ 69 folds). Among kinases involved in cellular processes, majority of kinases showed up regulation in PKSS with respect to PKS-15 (≤ 42 folds).

PKSS, growing in natural habitat is exposed to field conditions where everyday fluctuations in climatic conditions along with other unfavorable conditions are encountered whereas *in vitro* shoots (PKS-15 and PKS-25) are grown in a uniform and controlled environment. To identify kinases associated with P-I accumulation, this variation in growth conditions was minimized by analyzing expression of these kinases in high vs low P-I content genotypes (PKS-1- 2.7% and PKS-4- 0.3%) grown under green-house conditions. The analysis further shortlisted sixteen kinases, which were up regulated in high versus low P-I content genotype. As these shoots accumulate different P-I content in spite of growth in the same environmental conditions, up regulation of these kinases indicates their involvement in processes relevant for its biosynthesis. Final shortlisted kinases which showed association with P-I biosynthesis included MAP4K1, MAPK-3, 5, G-type lectin RLK, Ser/thr CX-32, Casein kinase II, PERK1, pto interacting kinase, UMP-CMP-3 kinase, Pyruvate kinase, Phosphofructokinase, Adenylate kinase-2, PEP carboxykinase, Hexokinase, Shaggy related kinase and adenylyl sulfate kinase (Fig.8).

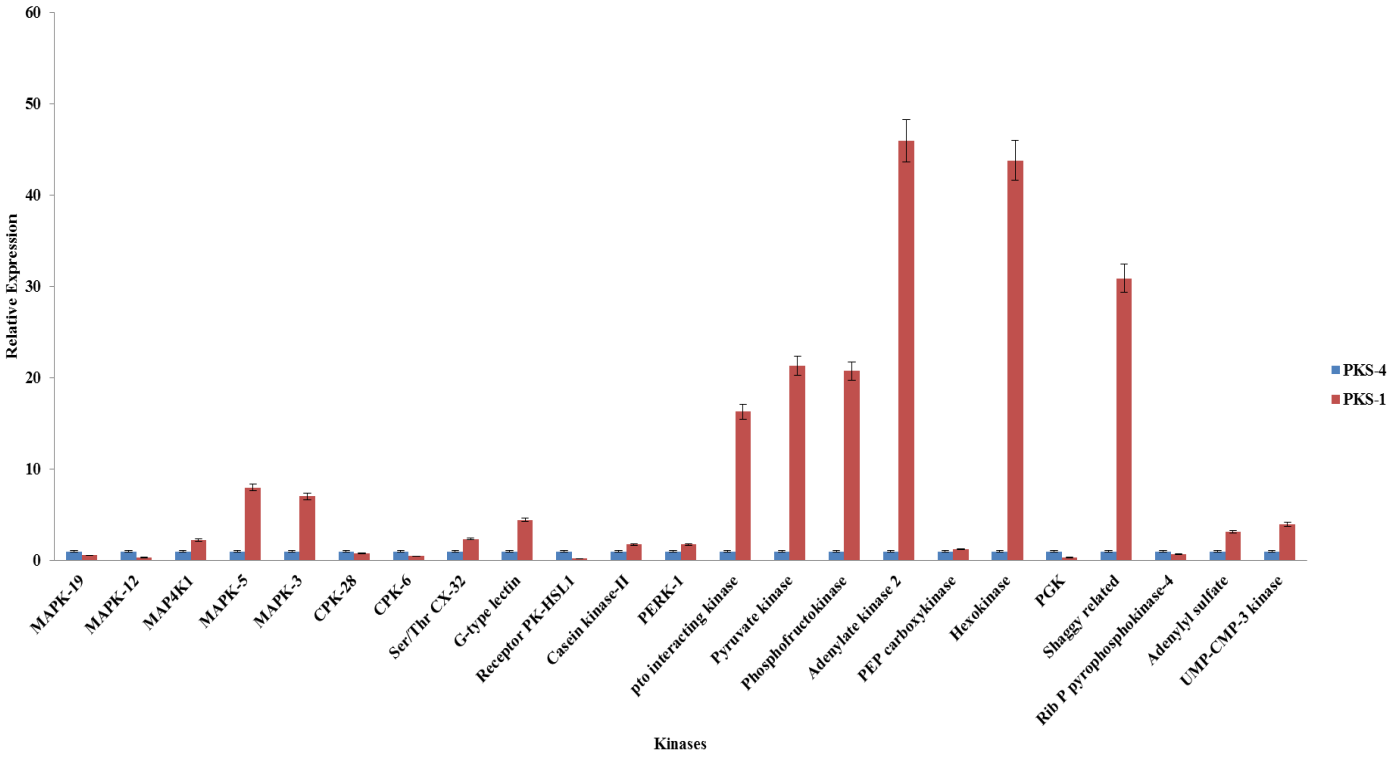

Figure 8. Relative expression of kinases in high (PKS-1- 2.7%) vs low (PKS-4- 0.3%) P-I content genotypes of *P. kurroa.* Error bars correspond to percentage error (5%)

Mapping kinases to physiological processes

Literature mining and KEGG database elucidated downstream processes affected under variable P-I accumulating conditions. The shortlisted kinases were observed to be implicated in numerous metabolic processes like light signaling, abiotic/biotic stress, biosynthesis of secondary metabolites, cell cycle progression, DNA damage control, nucleotide and carbohydrate metabolism, glycolysis/gluconeogenesis, photosynthesis, wounding, hormone response and sulfur metabolism. Metabolic processes found commonly associated with most of these kinases included abiotic/biotic stress, nucleotide and carbohydrate metabolism, wounding, hormonal response and biosynthesis of secondary metabolites (Fig.9) which highlights their role in affecting biosynthetic machinery of P-I in *P. kurroa*. Simultaneous up regulation of these processes in high P-I accumulating conditions indicates that when these pathways are induced, P-I production is triggered in *P. kurroa*.

Among the various classes of kinases analyzed for their role in P-I accumulation, a predominant role of kinases involved in cellular processes was indicated as majority of these kinases showed expression correlation with P-I content. Primary biosynthetic pathways like nucleotide and amino acid metabolism, glycolysis and gluconeogenesis, C-fixation and photosynthesis are producers of precursors like Glyceraldehyde-3-phosphate, Acetyl coA, PEP, Erythrose-4-

phosphate and Pyruvate which are the initiating points for biosynthesis of picrosides in *P. kurroa* (Fig. 1). Relevance of Pyruvate kinase and Hexokinase has been previously studied in relation to P-I biosynthesis where a collaboration of primary and secondary biosynthetic pathways was established in *P. kurroa* [35]. Pyruvate kinase, Hexokinase and Phosphofructokinase are regulatory enzymes of the glycolytic pathway [36] which is the central pathway providing precursors for shikimate pathway, mevalonate and non-mevalonate pathway, which have been reported to be contributing pathways for P-I biosynthesis [7]. Expression correlation of these genes with P-I content indicates availability of larger precursor pool in high P-I accumulating conditions.

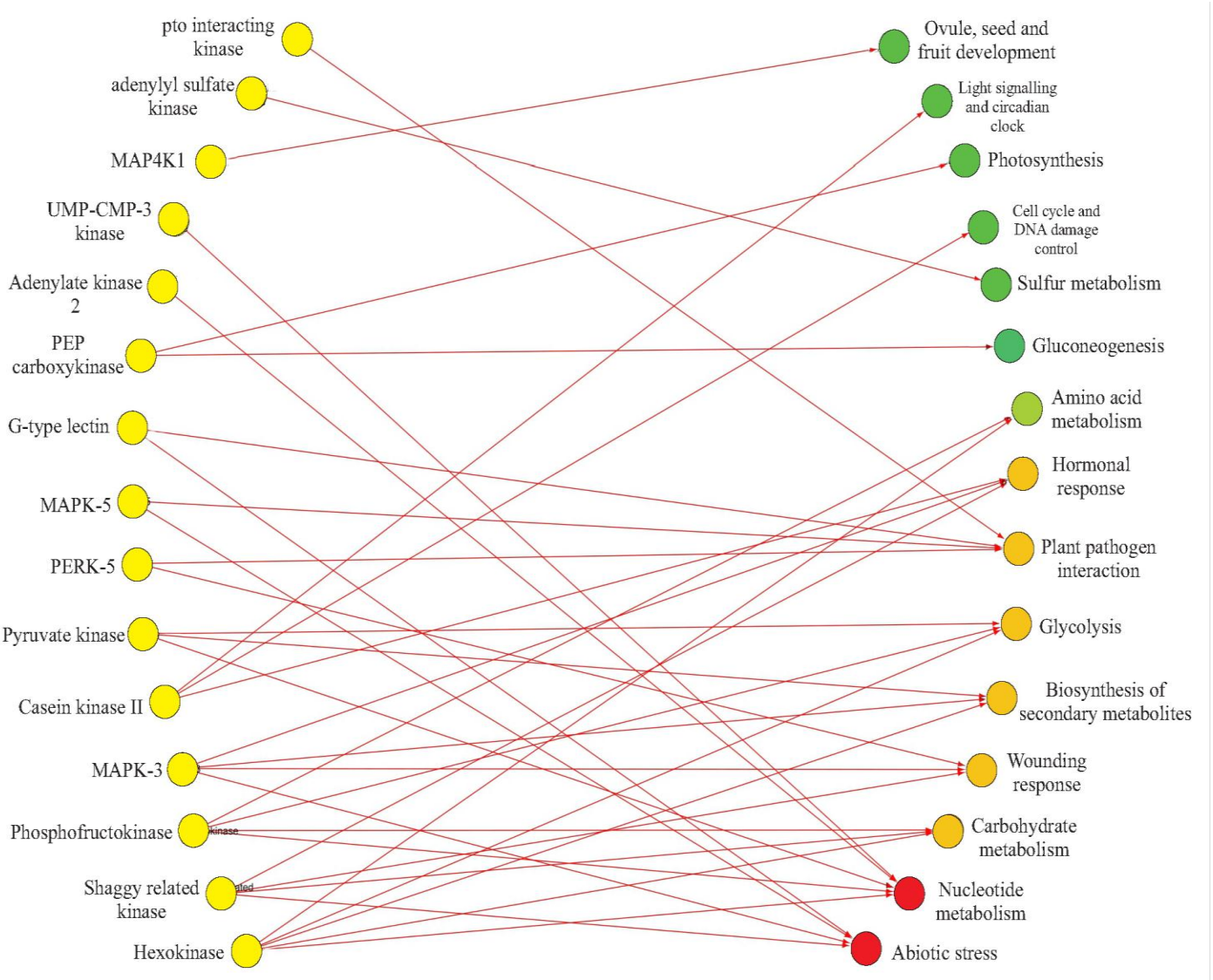

Figure 9. A network connectivity diagram representing molecular pathways associated with the shortlisted kinases showing correlation with picroside-I content

Genes affecting shoot biomass productivity in tissue cultured shoots of *P. kurroa*

Among tissue culture variants (PKS-25 and PKS-15), ~ 2 fold variation in shoot biomass has been observed [16]. This variation was utilized for identifying differentially expressed kinases in PKS-15 vs PKS-25 which might be affecting shoot biomass productivity in *P. kurroa*. Two kinases, CPK-28 and PERK1 have been linked with stem elongation and shoot production in Arabidopsis [37, 38] and their ~ 2 fold up regulation in PKS-15 vs PKS-25 indicates their possible involvement in eliciting shoot biomass in shoot cultures of *P. kurroa*. RLK Feronia and MAPK-12 which have been reported to be associated with auxin promoted growth [39, 40] were also found to be up regulated in PKS-15 vs PKS-25.

To get a deeper insight into molecular basis of shoot biomass variation, several genes known for their role in affecting shoot biomass either via hormonal response, leaf morphology or by transcriptional regulation were analyzed across these shoot tissue variants. *AXR-1, GRF-2, AVP-1, CLAVATA-1, TOR-1, AUX-1, LAX, DA-1* and *ARF-2* genes were found in both tissue cultured shoots and their *in silico* transcript abundance was observed to be higher in PKS-15 as compared to PKS-25. qRT-PCR analysis of these genes showed significant up regulation of *AXR-1, AVP-1, CLAVATA-1, TOR-1, AUX-1* and *LAX* in PKS-15 vs PKS-25 (> 2 folds) (Fig. 10).

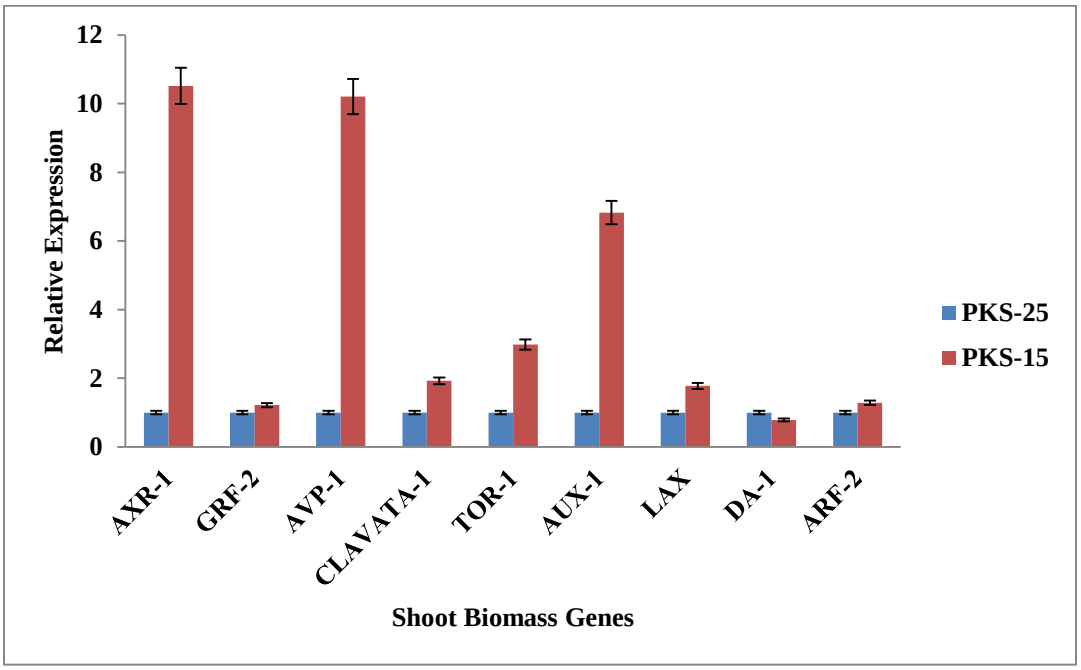

Figure 10. Relative expression of shoot biomass genes between high versus low shoot biomass variants of *P. kurroa* (PKS-15 vs PKS-25). Error bars correspond to percentage error (5%)

Previous studies have also emphasized the role of *AXR-1, AUX-1 and LAX* in controlling auxin signaling and responses, thereby affecting shoot development and branching [41, 42, 43]. AVP1 has been linked to regulation of leaf size [44] and as leaves of PKS-15 are larger in size as

compared to PKS-25, its role in regulation of leaf size in *P. kurroa* is expected. In our study, several genes which were found to be up regulated in PKS-15 as compared to PKS-25 in relation to shoot biomass (*AXR-1, AUX-1, LAX*, Feronia and MAPK-12) were observed to be auxin responsive. Moreover, the culture media which has been standardized for optimum growth of *P. kurroa* plants under *in vitro* conditions has a high auxin to cytokinin ratio (3 IBA: 1 Kinetin) [5]. Higher expression of auxin responsive genes in PKS-15 might be involved in better uptake and utilization of auxins from the culture media resulting in better shoot biomass productivity. High temperature injury has been reported to cause reduction in auxin content in Arabidopsis and *Hordeum vulgare* [45] and as shoot biomass in *P. kurroa* gets significantly reduced when grown under 25° C as compared to 15° C, a similar phenomenon of high temperature mediated effect on auxin signaling might be involved. The key genes identified for their role in affecting shoot biomass can be utilized as candidates for planning genetic engineering approaches aimed at enhancing shoot biomass in tissue culture conditions and also in natural habitat.

Identification of causal agent of leaf blight in *P. kurroa*

During sampling, leaf blight was often observed in field grown plants of *P. kurroa*. On the basis of morphological characteristics of conidiophores and conidia, the pathogen causing leaf blight in *P. kurroa* was identified as *Alternaria* sp (Fig. 11). ITS1/4 portion (402 bp) amplified from mycelial DNA showed 100% similarity with *Alternaria tenuissima* GenBank Accession No. KM921667.1. Nucleotide sequence of isolated ITS 1/4 region was submitted to GenBank (KT823704). Amplicons of species specific primers corresponding to *A. tenuissima* histone (CNL01), Hrip1, and LEU2 genes showed 99% similarity to *A. tenuissima* accessions EF371552.1, HQ713431.1, and EU294180.1, respectively. Inoculation of healthy *P. kurroa* leaves with fungal spores resulted in spots similar to infected field plants after 72 hrs whereas, control leaves remained asymptomatic (Fig. 11). As leaves are a major source of P-I in *P. kurroa*, fungal infestations can cause significant reductions in leaf biomass which can affect supply of quality raw material for herbal preparations. Proper identification of this pathogen would facilitate investigation of sustainable disease control in this pathosystem and development of efficient large scale cultivation methods for *P. kurroa*. The study reported the causal pathogen of leaf blight for the first time in *P. kurroa*.

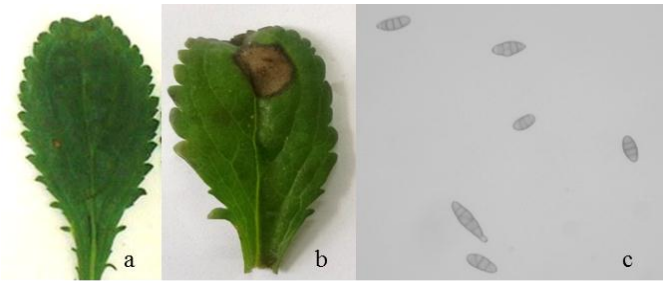

Figure 11 Healthy leaf of *Picrorhiza kurroa* (a) Infected leaf (b) Spore morphology of fungal culture (400x) (c)

Conclusion and future prospects

The outcome of the research work is expected help in understanding the complex regulatory mechanisms governing picrosides accumulation in *P. kurroa*. Regulation of picrosides biosynthetic pathway genes was explored via analysis of important molecular regulators like transcription factors and miRNAs. miR-4995 was found to have a potential role in down-regulation of P-I biosynthesis in tissue cultured shoots. It was observed to target DAHPS enzyme, which is regulatory step of shikimic acid pathway, a major contributing pathway for picrosides biosynthesis. Silencing of miR-4995 can possibly result in enhanced P-I production in tissue cultured shoots of *P. kurroa* which is a major pre-requisite for fulfilling industrial demands via tissue culture. Further, transcription factors like ERF-18, NAC-25, WRKY-40 and MYB-4 were observed to target cis-regulatory elements of key genes of picrosides biosynthetic pathway, indicating their significant role in regulation of picrosides production in *P. kurroa*. It was observed that WRKY-40 and MYB-4 might be involved in repression of these genes, resulting in low picrosides production in tissue cultured plants of *P. kurroa*. Functional analysis of these key TFs will provide an important foundation for planning a combined approach involving pathway enzymes and TFs which can lead to optimal flux levels in pathway leading to enhanced metabolite content in *P. kurroa*. Study of kinases involved in signal transduction cascades provided an insight into primary metabolic processes stimulated by environmental cues, affecting variable P-I production in shoot tissues of *P. kurroa*. Elevated expression of *AXR-1*, *AUX-1*, *LAX*, *Feronia* and *MAPK-12* in high shoot biomass accumulating shoots indicated a significant role of auxin response in regulation of shoot biomass productivity in *P. kurroa*. These genes can be taken up as candidates for overexpression studies aimed at enhancing shoot biomass yield, thereby leading to higher P-I production. Overall, the current work has uncovered several aspects associated with regulation of picrosides biosynthesis in *P. kurroa*. The research work provides several leads for planning genetic interventions aimed at enhancing picrosides production in *P. kurroa*.

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LIST OF PUBLICATIONS

[1] **Vashisht I**, Mishra P, Pal T, Chanumolu S, Singh TR and Chauhan RS (2015) Mining NGS transcriptomes for miRNAs and dissecting their role in regulating growth, development, and secondary metabolites production in different organs of a medicinal herb, *Picrorhiza kurroa*. *Planta* 241(5): 1255-68 **(I.F.: 3.24; SCOPUS)**

[2] **Vashisht I** and Chauhan RS (2016) First Report of Leaf Spot on a Medicinal Herb, *Picrorhiza kurroa* (Royle ex. Benth) Caused by *Alternaria tenuissima* in India. *Plant Disease* 100(3): 647 **(I.F.: 3.19; SCOPUS)**

[3] **Vashisht I**, Pal T, Sood H, Chauhan RS (2016) Differential Metabolites Content Transcriptomes Pinpoint Key Transcription Factors Regulating Picrosides Biosynthesis in a Medicinal Herb, *Picrorhiza kurroa* Royle ex. Benth. *Molecular Biology Reports* 43 (12): 1395-1409 **(I.F.: 1.69; SCOPUS)**

[4] **Vashisht I**, Pal T, Bansal A and Chauhan RS. Uncovering interconnections between kinases vis-à-vis physiological and biochemical processes contributing to picroside-I biosynthesis in a medicinal herb, *Picrorhiza kurroa* Royle ex. Benth. *Acta Physiolgiae Plantarum* (Communicated). Manuscript ID: ACPP-D-16-01312 **(I.F.: 1.56; SCOPUS)**

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[1] **Vashisht I**, Chanumolu S, Pal T, Mishra P, Singh TR and Chauhan RS. Deciphering the Role of miRNAs in Regulating Growth, Development and Secondary Metabolites Production in an Endangered Medicinal Herb, *Picrorhiza kurroa*. 7th Annual Convention of ABAP & International Conference on Plant Biotechnology, Molecular Medicine & Human Health, Delhi University, South Campus, Delhi, October 18-20, 2013.

[2] **Vashisht I**, Pal T and Chauhan RS**.** Mining *Picrorhiza kurroa* transcriptomes for transcription factors contributing to picrosides biosynthesis. 3rd International Plant Physiology Congress, Jawaharlal Nehru University, New Delhi, December 11-14, 2015.

NCBI GENBANK SUBMISSION

Vashisht I and Chauhan RS (2016)
Alternaria tenuissima strain APk-1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence, KT823704.